Cell, Vol. 77, 1117–1124, July 1, 1994, Copyright © 1994 by Cell Press

Identification and Cloning of a Megakaryocyte Growth and Development Factor That Is a Ligand for the Cytokine Receptor Mpl

T. D. Bartley,* J. Bogenberger,* P. Hunt,* Y.-S. Li,* H. S. Lu, F. Martin, M.-S. Chang, B. Samal, J. L. Nichol, S. Swift, M. J. Johnson, R.-Y. Hsu, V. P. Parker, S. Suggs, J. D. Skrine, L. A. Merewether, C. Clogston, E. Hsu, M. M. Hokom, A. Hornkohl, E. Choi, M. Pangelinan, Y. Sun, V. Mar, J. McNinch, L. Simonet, F.. Jacobsen, C. Xie, J. Shutter, H. Chute, R. Basu, L. Selander, D. Trollinger, L. Sieu, D. Padilla, G. Trail, G. Elliott, R. Izumi, T. Covey, J. Crouse, A. Garcia, W. Xu, J. Del Castillo, J. Biron, S. Cole, M. C.-T. Hu, R. Pacifici, I. Ponting, C. Saris, D. Wen, Y. P. Yung, H. Lin, and R. A. Bosselman
Amgen, Incorporated
Amgen Center
Thousand Oaks, California 91320

Summary

A novel megakaryocyte growth and development factor (MGDF) has been identified in aplastic canine plasma, and its cDNAs have been cloned from canine, murine, and human sources. Purified canine MGDF isolated by procedures involving Mpl receptor affinity chromatography exists in at least two forms, with apparent molecular masses of 25 kDa and 31 kDa, that share the N-terminal amino acid sequence APPACDPRLLNKMLRDSHVLH. Human, dog, and mouse cDNAs for MGDF are highly conserved and encode open reading frames for proteins of 353, 352, and 356 amino acids, respectively, including predicted signal peptides. Canine MGDF and recombinant human MGDF support the development of megakaryocytes from human CD34+ progenitor cell populations in liquid culture and promote the survival of a factor-dependent murine cell line (32D) engineered to express Mpl. These biological activities are blocked by the soluble extracellular domain of Mpl. These data demonstrate that MGDF is a novel cytokine that regulates megakaryocyte development and is a ligand for the MPl receptor.

Introduction

Megakaryopoiesis and platelet production are vital to mechanisms of homeostasis, including storage and release of cytokines, wound healing, and blood coagulation. The failure of an organism to maintain adequate megakaryocyte numbers leads to thrombocytopenia and consequent bleeding disorders that can, in the extreme, r sult in death. The humoral factors that physiologically regulate megakaryocyt and platelet d velopment ar as y t unknown. Although s veral cytokines hav influences on megakaryocyte development, including interleukin-1 (IL-1) (Schmidt, 1984; March et al., 1985), IL-3 (Yang et al., 1986; Ikebuchi et al., 1987), IL-6 (Hirano et al., 1985, 1986; Ishibashi et al., 1989), IL-11 (Paul et al., 1990; Teramura et al., 1992), leukemia inhibitory factor (Metcalf et al., 1991), granulocyte–macrophage colony-stimulating factor (Wong et al., 1985), erythropoietin (Miyake et al., 1977; Jacobs et al., 1985), and stem cell factor (Hendrie et al., 1991), megakaryopoiesis does not appear to be their primary function. Additional activities implicated in megakaryopoiesis include megakaryocyte-potentiating factor (Yamaguchi et al., 1994), megakaryocyte stimulatory factor (Tayrien and Rosenberg, 1987; Greenberg et al., 1987), megakaryocyte colony-stimulating factor (Ogata et al., 1990; Erickson-Miller et al., 1992, 1993), thrombopoiesis-stimulating factor (McDonald et al., 1975), and thrombopoietin (Hill and Levin, 1986; Hill et al., 1992). Sources for these activities have included the urine, serum, or plasma from aplastic and/or thrombocytopenic humans (McDonald, 1975; Ogata et al., 1990), rats (Odell et al., 1961), rabbits (Evatt et al., 1974; Hill et al., 1992), and dogs (Mazur and South, 1985). However, the low initial concentrations of these activities, as well as the complexity of these sources, have prevented the unambiguous identification of a lineage-specific cytokine(s) for megakaryocytes or platelets.

Studies of the cytokine receptor Mpl have suggested that this protein has a role in megakaryopoiesis. A portion of the gene for this receptor was originally identified in the myeloproliferative leukemia virus as the oncogene v-*mpl* (Souyri et al., 1990). Myeloproliferative leukemia virus causes a broad spectrum of mammalian leukemias, including erythroid, granulocytic, monocytic, megakaryocytic, and mast cell leukemias (Wendling et al., 1986, 1989). Subsequent cloning of c-*mpl* (Vignon et al., 1992; Skoda et al., 1993) confirmed its identity as a member of the hematopoietin receptor superfamily (Bazan, 1990), a family characterized by a common structural design of the extracellular domain, including four conserved C residues in the N-terminal portion and a WSXWS motif close to the transmembrane region. Evidence that c-*mpl* plays a functional role in hematopoiesis includes observations that its expression is restricted to spleen, bone marrow, or fetal liver in mice (Souyri et al., 1990) and to megakaryocytes, platelets, and CD34+ cells in humans (Methia et al., 1993). Furthermore, exposure of CD34+ cells to synthetic oligodeoxynucleotides antisense to *mpl* mRNA significantly inhibits the appearance of megakaryocyte colonies without affecting erythroid or myeloid colony formation (Methia et al., 1993). These observations suggest that Mpl is a cell surface receptor for a cytokine that regulates megakaryopoi sis.

This r port describes a megakaryocyte growth and development factor (MGDF) pres nt in aplastic canine plasma, its N-terminal amino acid s qu nc , the isolation of cDNA clones from dog and mouse, and human cDNA clones that express MGDF activity. MGDF binds to Mpl affinity columns, and its biological effects ar inhibit d by the soluble extracellular domain of Mpl (Mpl-X). These

*These four authors contributed equally to this work.

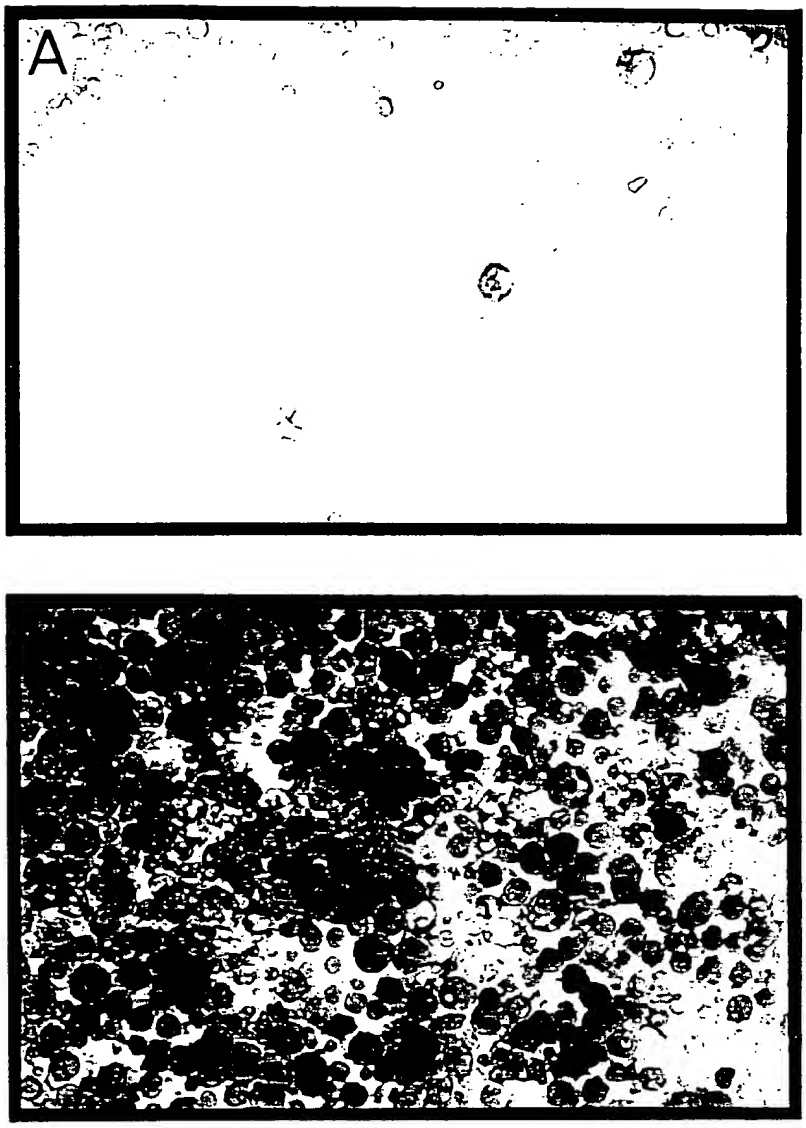

Figure 1. Aplastic, but Not Normal, Canine Plasma Induces the Growth and Development of Human Megakaryocytes from CD34+ Cells

CD34-selected cells (3000 cells/well) were plated in the presence of 10% normal canine plasma (A) or 10% aplastic canine plasma (APK9) (B). After 8 days in culture, the cells were fixed and stained for megakaryocytes in situ. Megakaryocytes are identified by the blue color reaction.

data indicate that MGDF plays an important role in normal megakaryocyte biology and is a ligand for the cytokine receptor Mpl.

Results and Discussion

The Megakaryocyte Growth and Development Activity within Aplastic Canine Plasma Interacts with Mpl

The in vitro growth and development of human megakaryocytes from CD34+ peripheral blood cells is promoted by aplastic canine plasma (APK9) (Nichol et al., submitted). APK9 is maximally active when collected 10–14 days after myeloablative irradiation, at which time the platelet count is 1%–6% of normal (data not shown). Figure 1 displays CD34+ cells incubated for 8 days in 10% normal canin plasma (Figure 1A) or in 10% APK9 (Figure 1B). While th c lls cultur d in normal plasma fail to thriv , cells cultur d in APK9 develop almost xclusiv ly into megakaryocytes. This ff ct of APK9, also obs rved in human marrow (Mazur and South, 1985) or peripheral blood leukocytes (Mazur et al., 1990), cannot be duplicated with IL-3, IL-6, IL-11, granulocyte–macrophage colony-stimulating factor, leukemia inhibitory factor, stem cell factor, or erythropoietin and is attributed to another (presently unknown) factor(s) (Mazur and South, 1985; Mazur et al., 1990; Nichol et al., submitted). We examined whether this factor(s) was a protein(s) that interacts with Mpl. When the extracellular portion of the murine receptor (Mpl-X) was added to cultures of APK9-stimulated CD34+ cells, megakaryocyte development was completely inhibited. Inhibition was dose-dependent and complete at 5 μg/ml (Figure 2). In contrast, Mpl-X at 10 μg/ml had no inhibitory ffect on IL-3- or granulocyte–macrophage colony-stimulating factor–induced cell growth or on erythropoietin plus IL-3–induc d erythroid development (data not shown). Thes data, lik those of Methia et al. (1993), imply that Mpl is involved in m gakaryocyt but not myeloid or rythroid development and that MGDF functions through an interaction with this receptor. Further vidence that APK9 contains proteins

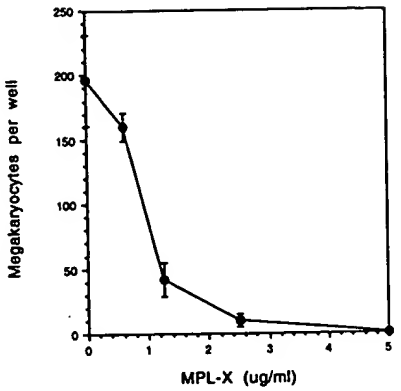

Figure 2. The Megakaryocyte Growth and Development Activity within APK9 Is Inhibited with Mpl-X

CD34-selected cells (3000 cells/well) were incubated in 5% APK9 and increasing concentrations of Mpl-X. After 8 days in culture, the cells were fixed and stained for megakaryocytes in situ. The megakaryocytes were enumerated from each of triplicate wells. Data are presented as the mean number of megakaryocytes per well ± SEM.

that interact with Mpl was provided by experiments using a factor-dependent (IL-3) cell line engineered to express murine Mpl (32D/Mpl$^+$). APK9, but not normal canine plasma, supported the survival and growth of this cell line. This activity, not attributable to IL-3, was blocked by Mpl-X in a dose-dependent manner (data not shown). On the basis of these observations, we designed a purification scheme, centered on an Mpl-X receptor affinity column, to isolate MGDF from APK9.

N-Terminal Sequence of Purified Canine MGDF

Canine MGDF was purified approximately 10^7-fold from APK9 by a purification scheme based upon Mpl-X affinity chromatography in conjunction with wheat germ agglutinin, ion exchange, gel filtration, and reverse-phase high pressure liquid chromatography. Details of the purification will be presented elsewhere. Two species of purified MGDF were observed, with apparent molecular masses of 25 kDa and 31 kDa. Figure 3 clearly demonstrates that the biological activity observed coincides with these two isolated proteins, as determined by comigration on an SDS–polyacrylamide gel. Both protein species generated mature megakaryocytes in liquid culture, supported the survival of 32D/Mpl$^+$ cells (but not the parental 32D cells), and were equivalently inhibited with Mpl-X. These results demonstrate that MGDF is the protein that promotes survival of 32D/Mpl$^+$ cells and is a ligand for Mpl.

N-terminal sequence analysis of the purified 25 kDa and 31 kDa proteins yielded the same amino acid sequence, APPA(X)DPRLLNKMLRDSHVLH. A computer-based homology search (Devereux et al., 1984) sh wed that this sequence was novel.

Cl ning f cDNA Coding f r MGDF and Sequ nce Comparison f Human, Canin , and Murine Speci s

Degenerate oligonucleotides, based on the amino acid sequence obtained from th N-t rminus of purified canin

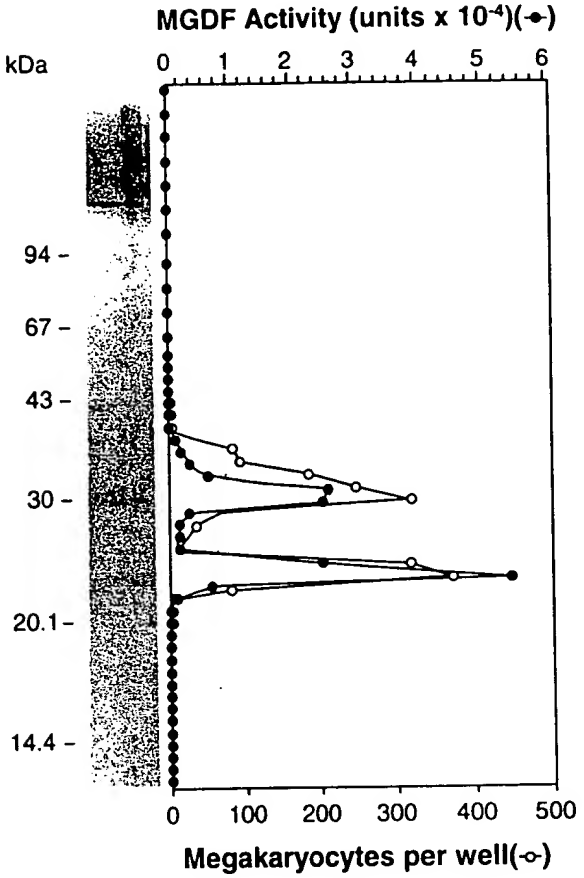

Figure 3. Purified Canine MGDF Was Separated by SDS–Polyacrylamide Gel Electrophoresis

One lane of the gel was silver stained, and the adjacent lane was sliced into 1 mm sections. The gel pieces were extracted overnight with phosphate-buffered saline with 5 mM CHAPS. The diafiltered supernatants were assayed for activity on 32D/Mpl$^+$ cells (closed circles) and on CD34$^+$ cells (open circles).

MGDF, were designed for polymerase chain reaction (PCR) amplification of specific DNA segments from canine kidney cDNA. A DNA fragment was isolated that encoded the expected canine N-terminal sequence. This PCR product was used as a probe to screen a human fetal liver cDNA library in a cytomegalovirus promoter-based mammalian expression vector. A full-length cDNA clone containing a 1.35 kb insert was isolated and further analyzed (Figure 4). This cDNA clone encoded an open reading frame of 353 amino acids that had a region of high homology to the N-terminal sequence of canine MGDF. Sequences for canine and murine MGDF were obtained by PCR amplification of cDNA from adult kidney or liver RNA, respectively.

The deduced amino acid sequences of human, canine, and murine MGDF were compared (Figure 5). The proteins of all three species are highly homologous, exhibiting 69.1%–76.5% identity (77.6%–83.0% similarity). Th N-terminal two thirds of MGDF is more highly cons rved than the r mainder of the molecul . A search of computer data bases (Devereux et al., 1984) show d that MGDF is most

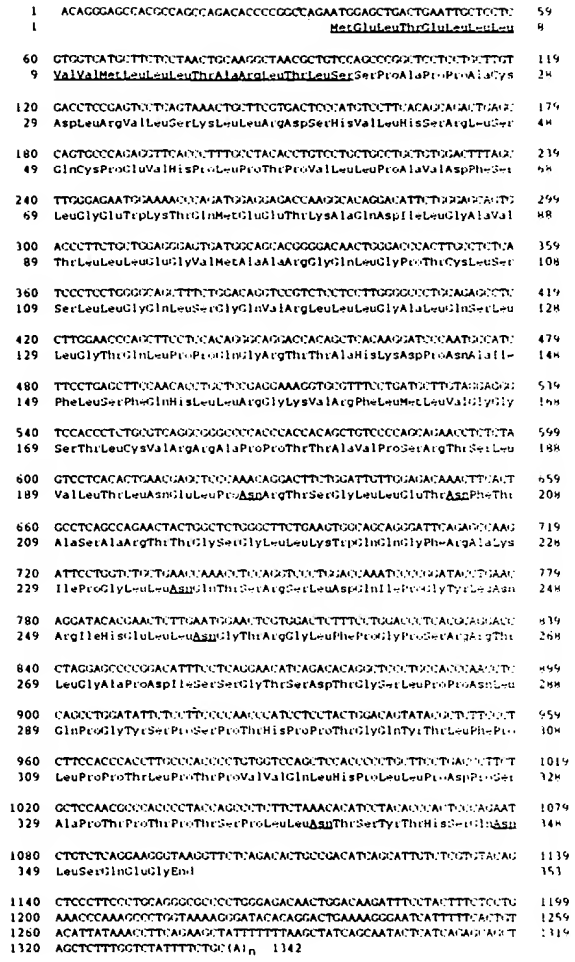

Figure 4. Nucleotide Sequence of a cDNA Coding for Human MGDF and Deduced Amino Acid Sequence

The amino acid sequence of an open reading frame of 353 residues starting at position 32 and ending at position 1098 is shown. A predicted signal peptide of 21 amino acids is underlined (von Heijne, 1986). Potential N-linked glycolsylation sites at Asn-197, Asn-206, Asn-234, Asn-255, Asn-340, and Asn-348 are underlined.

closely related to erythropoietin (25% identity). Homology is confined to the N-terminus (amino acids 1–172). The fact that the C-terminus of MGDF is less conserved may hav functional implications.

The 25 kDa and 31 kDa forms of MGDF, purified from APK9, are smaller than the predicted unglycosylated 35

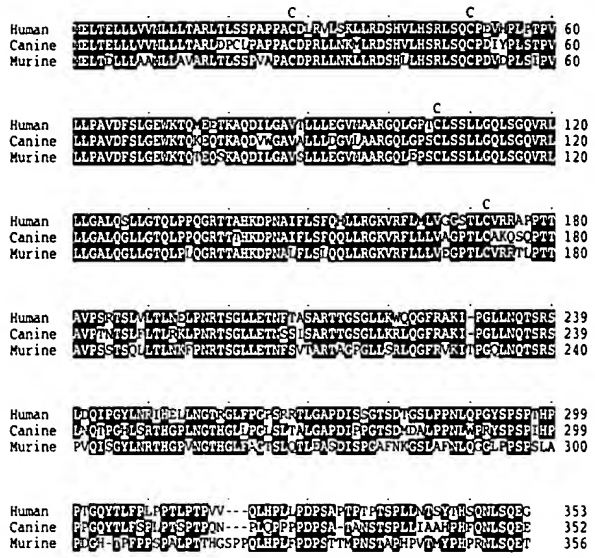

Figure 5. Alignment of Deduced Amino Acid Sequences from Human, Canine, and Murine cDNAs Coding for MGDF

Identical amino acids present in at least two species are in white on black background. The four conserved cysteines found in all species are highlighted.

kDa primary translation product. Since these forms share the same N-terminus, they may be the products of proteolytic processing at the C-terminus. This interpretation is consistent with the fact that the C-terminus is less conserved between species and that expression of a truncated human cDNA, encoding amino acids 1–195, yields a biologically active protein (see below).

Biological Characterization of Recombinant Human MGDF

Human MGDF cDNA in the vector pBCB was transfected into 293 EBNA cells in order to express recombinant human MGDF (rhMGDF). Conditioned medium was collected at 72 hr posttransfection. Biological activity of rhMGDF was detected in the same bioassays originally used to detect natural canine MGDF. 32D cells expressing either murine or human c-*mpl* survived without IL-3 when grown in conditioned medium from cells transfected with the human MGDF clone (Table 1). Untransfected 293 EBNA cells did not produce this activity. The parental 32D

Table 1. Biological Activity of rhMGDF

MGDF cDNA Transfected	Mpl-X Added	Activity on 32D Cells (U/ml)		
		32D	32D/mu-*mpl*	32D/hu-*mpl*
Full length (1–353 amino acids)	–	0	25,600	51,200
	+	–	1,600	1,600
Truncated (1–195 amino acids)	–	0	25,600	25,600
	+	–	1,600	3,200

Conditioned media from 293 EBNA cells transfected with cDNA coding for rhMGDF were added to 32D cells, expressing either murine (mu) or human (hu) *mpl*. Species-specific inhibition was demonstrated by the addition of the corresponding Mpl-X to 10 μg/ml.

Table 2. rhMGDF Induces the Generation of Human Megakaryoctes

Factor Source	Megakaryoctes per Well	
	Without Mpl-X	With Mpl-X
APK9	100 ± 3	0
HuAA	101 ± 18	0
rhMGDF (1–353 amino acids)	142 ± 48	17 ± 2
rhMGDF (1–195 amino acids)	100 ± 3	6 ± 2
Vector control	0	0

Conditioned media from 293 EBNA cells transfected with cDNA coding for rhMGDF were added to CD34⁺ cells plated as described in the legend to Figure 2. Conditioned medium was used at 20% final volume. APK9 plasma and human aplastic anemia (HuAA) patient sera were used at 6% and 10% final volume, respectively. Mpl-X was added to a final concentration of 30 μg/ml.

cells that did not express the c-*mpl* did not survive in the absence of IL-3 either with or without conditioned medium from rhMGDF-transfected 293 EBNA cells. The activity of rhMGDF was inhibited by murine or human Mpl-X, corroborating the original observations on natural canine MGDF and confirming the role of Mpl in the action of MGDF. Similar results were obtained with a human MGDF cDNA artificially truncated after Leu-195.

Conditioned medium containing either the full-length or truncated form of rhMGDF also induced megakaryocyte development from human CD34⁺ cells. Megakaryocyte development was comparable to that achieved with either APK9 or human aplastic anemia patient sera (courtesy of Dr. R. Paquette, University of California, Los Angeles). In all cases, megakaryocyte development was substantially inhibited with Mpl-X at 30 μg/ml (Table 2). In other experiments (data not shown), inhibition was complete with 100 μg/ml Mpl-X. The kinetics of appearance of megakaryocytes was similar to that observed with APK9 (data not shown). Cell populations induced by rhMGDF were predominantly megakaryocytic (Figure 6). After 8 days of culture in the presence of either the full-length form or the truncated form of rhMGDF, 37% or 41% of the respective populations were mature megakaryocytes displaying platelet glycoproteins. The remainder of the cells were unidentified blast-like cells, macrophages, or small mononuclear cells. Populations of megakaryocytes present in these cultures were phenotypically normal, exhibiting the four maturation stages characteristic of this lineage.

These observations confirm that cDNAs encoding rhMGDF express biologically active protein analogous to that isolated from canine aplastic plasma and show that MGDF exhibits the biological properties of a ligand for Mpl. MGDF is necessary and sufficient to induce in vitro megakaryocyte development in the presence of normal human plasma and therefore is likely to play an essential role in the regulation of this cell lineage. Further characterization of MGDF will provide insights into megakaryocyte biology and could lead to important th rapeutic applications for the treatment of thrombocytopenia associated with various clinical settings such as bone marrow transplantation, irradiation, and chemotherapy of cancer.

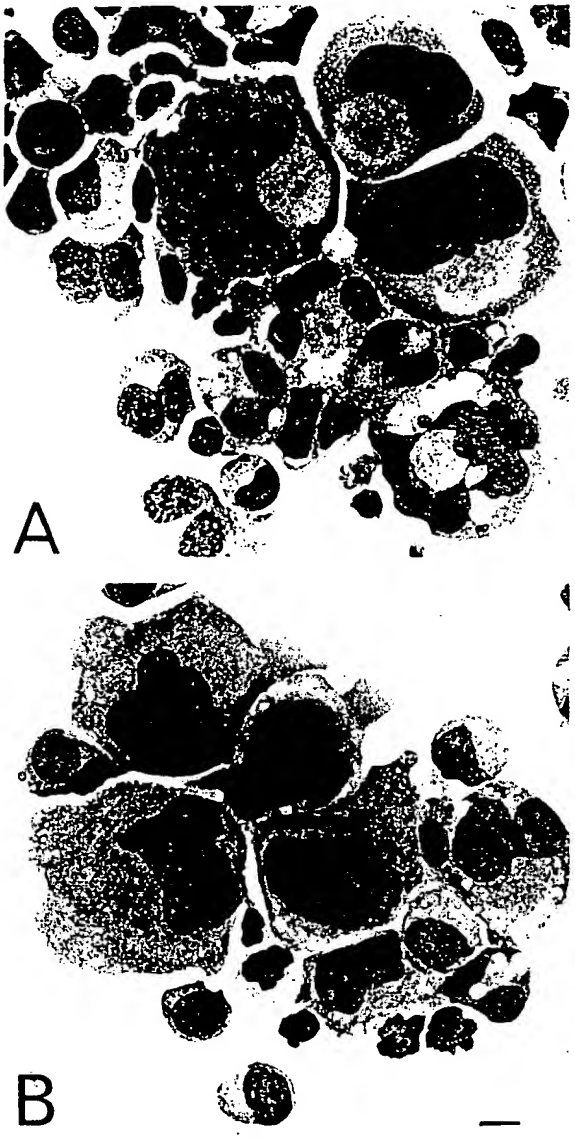

Figure 6. rhMGDF Induces the Growth and Development of Human Megakaryocytes from CD34⁺ Cells

CD34-selected cells were cultured in 5% conditioned media from EBNA 293 cells transfected with full-length MGDF cDNA (A) or truncated MGDF cDNA (amino acids 1–195) (B). After 8 days in culture, the cells were harvested, cytocentrifuged, stained, and photographed as described in Experimental Procedures. Scale bar, 10 μm.

Experimental Procedures

Assays for Human Megakaryocyte Progenitors

Normal heparinized platelet-poor AB plasma was obtained from healthy adult volunteers with informed consent. Leukapheresis units were purchased from Hemacare (Sherman Oaks, CA). Donors provided informed consent and were from a pool of repeat donors committed to the program. Heparinized plasma from aplastic dogs was produced as described by Mazur and South (1985), except that 450 rads of total body irradiation were delivered to each animal. Plasma was stored frozen until use.

CD34-selected cells from human leukapheresis units were tested for megakaryocyte-generating potential in a liquid culture assay as described elsewhere (Nichol et al., submitted). In brief, cells from leukapheresis units were enriched for megakaryocyte progenitors by elu-

triation and immunomagnetic CD34 selection (Miltenyi Biotec, Sunnyvale, CA). $CD34^+$ cells were plated at 3000 cells (15 μl final volume) in the indicated concentration of growth factor in wells of Terasaki-style microtiter plates (Vanguard, Incorporated, Neptune, NJ). Medium was Iscove's modified Dulbecco's medium (GIBCO, Grand Island, NY), supplemented as described elsewhere (Nichol et al., submitted), including 10% normal, heparinized, and platelet-poor human AB plasma. Cells were incubated at 37°C for 8 days in humidified boxes in 5% CO_2 in air, fixed directly to the culture wells with 1% glutaraldehyde, and incubated with a monoclonal antibody cocktail (anti-GPIb and anti-GPIIb; BioDesign, Kennebunkport, ME) and anti-GPIb (Dako, Carpinteria, CA). The immune reaction was developed with a streptavidin–β-galactosidase detection system (HistoMark, Kirkegaard & Perry Laboratories, Gaithersburg, MD). Megakaryocytes, identified by the blue color, were enumerated with an inverted phase microscope at 100× magnification (Olympus, McBain Instruments, Chatsworth, CA). Results are presented as mean ± SEM.

For photography, cells were cultured at 3×10^5/ml (150 μl total volume) in flat-bottomed 96-well culture plates (Falcon 3072, Becton Dickinson, Oxnard, CA) for 8 days, cytocentrifuged onto glass slides (Cytospin, Shandon, Pittsburgh, PA), and stained by a modified Wright–Giemsa method in an Aerospray slide stainer (Wescor, Logan, UT). Slides were photographed with the Microphot-FXA microphotography system (Nikon, Melville, NY). Color video prints (Sony, San Jose, CA) of culture wells were obtained through an inverted microscope-mounted camera (Optronics LX-450, McBain Instruments, Chatsworth, CA).

Generation of Anti-Peptide Antibodies

A synthetic peptide (CGPTYQGPWSAWSPPARV) corresponding to amino acids 119–135 of v-Mpl (Souyri et al., 1990) was coupled to keyhole limpet hemocyanin and used to immunize rabbits (Berkeley Antibody Company, Richmond, CA). Antisera from these rabbits were affinity purified using an immobilized peptide (Affi-Gel 10, Bio-Rad, Richmond, CA).

Production of Mpl-X

The 3′ end of a cDNA clone coding for the murine form of c-*mpl* (Skoda et al., 1993) was deleted at nucleotide 1425 by digestion with DsaI, and synthetic oligonucleotides corresponding to the sequence (5′-CACGGGCTCCGAGACTGCTTGAGTCGACA-3′) were inserted. This procedure leads to replacement of Trp-483 with a stop codon (TGA). Proteins produced from this truncated clone represent the extracellular domain of murine Mpl (minus Trp-483). This DNA was inserted into the mammalian expression vector pDSR-α-2. CHO cells were transfected with this construct, selected, and amplified as previously described (DeClerck et al., 1991). The recombinant extracellular domain of murine Mpl (Mpl-X) was purified from CHO cell culture supernatants by a combination of ion exchange and hydroxylapatite chromatography. Human Mpl-X was produced and purified by identical procedures.

Mpl-Transfected, Factor-Dependent Cell (32D/Mpl^+) Assay

The full-length murine Mpl receptor sequence (Skoda et al., 1993) was subcloned into an expression vector containing a transcriptional promoter derived from the long terminal repeat of Moloney murine sarcoma virus. This contruct (5 μg) and the selectable marker plasmid pWLNeo (Stratagene) (1 μg) were coelectroporated into 4×10^6 IL-3-dependent 32D (clone 23) cells (Greenberger, 1983) in a 0.4 cm cuvette at 0.175 kV (1000 μF and 72 ohms). Cells were cultured for 5 days, divided into pools of 2×10^5 cells, and incubated in selective medium containing 800 μg/ml Geneticin (G418, Sigma, St. Louis, MO) and 1 ng/ml murine IL-3. Pools were tested for surface expression of Mpl receptor by FACS analysis using affinity-purified polyclonal rabbit anti-peptide sera, and cells expressing high levels of Mpl (32D/Mpl^+) were subsequently enriched by FACS using the same anti-peptide sera. Single-cell clones were further selected by growth in 10% aplastic canine plasma and Geneticin. After selection in aplastic canine plasma for 35 days, the cells were maintained in 1 ng/ml murine IL-3. 32D cells expressing human c-*mpl* were generated by infection with replication defective retroviral vector encoding full-length human c-*mpl* followed by FACS and growth selection in APK9 (Vignon et al., 1992; Landau and Littman, 1992). For the MGDF assay, 32D/Mpl^+ cells were washed free of culture IL-3 and replated (1000 cells; 15 μl total volume per well) in Terasaki-style microtiter plates (Vanguard, Neptune, NJ) in α-MEM (GIBCO, Grand Island, NY) supplemented with 10% fetal calf serum, Geneticin (800 μg/ml), and 1% penicillin/streptomycin (GIBCO, Grand Island, NY) in 1:1 serial dilutions of test samples. After 48 hr, the number of viable cells per well was determined microscopically. The amount of activity that resulted in 200 viable cells per well from an initial inoculum of 1000 cells per well was defined as 1 U of activity. Activity was attributed to MGDF only if it could be completely inhibited with 5–10 μg/ml Mpl-X. MGDF activity in APK9 averaged 4400 ± 539 U/ml. Recombinant murine IL-3 contained 1.6×10^9 U/mg when tested in the same assay. However, IL-3 was not inhibited with Mpl-X.

Gel Electrophoresis and Recovery of Purified MGDF

Electrophoresis was performed according to the method of Laemmli (1970), with the exception that reducing agent was omitted from the sample buffer. Precast gels were used (Novex, Encinitas, CA). Upon electrophoresis, the gel was sliced (1 mm) and the slices were diced with razor blades. The pieces were transferred to 1.5 ml microfuge tubes containing 0.5 ml phosphate-buffered saline, 5 mM CHAPS and gently agitated overnight at 4°C. The tubes were spun briefly, an aliquot was removed, and the sample was diafiltered for bioassay against Iscove's medium supplemented with 3 mg/ml BSA as a carrier protein.

N-Terminal Sequence Analysis of MGDF

Amino acid sequence determination was performed with a Model 473 protein sequencer (Applied Biosystems, Foster City, CA) equipped with a miniaturized sample cartridge and online phenylthiohydantoinyl amino acid analysis. Purified protein samples were loaded directly onto a glass fiber disc precycled with polybrene and NaCl. The phenylthiohydantoinyl amino acid analysis was performed with a microliquid chromatographic system (Model 120, Applied Biosystems, Foster City, CA) using dual syringe pumps and C18 narrow bore columns (2.1 × 250 mm, Applied Biosystems, Foster City, CA) using optimized elution conditions as recommended by the manufacturer. Data were collected and reprocessed using software (Model 610) from Applied Biosystems.

Cloning of Canine and Murine MGDF N-Terminal cDNAs

Degenerate oligonucleotide primers were designed based on the canine MGDF N-terminal amino acid sequence and used as primers in PCRs to amplify MGDF-encoding cDNA sequences. Total RNA was prepared from canine kidney samples by the guanidinium isothiocyanate method of Chomczynski and Sacchi (1987). First-strand cDNA was prepared with a random primer–adapter (5′-GGCCGGATAGGCCACTCNNNNNNT-3′) using Moloney murine sarcoma virus reverse transcriptase and was used as template in subsequent PCRs.

PCR was performed on 0.5 μl (about 50 ng) of the cDNA, using primer A (5′-GCNCCNCCNGCNTGYGA-3′), a sense-strand primer encoding amino acids 1–6 (APPACD), and either primer B (5′-GCARTGNAGNACRTGNGARTC-3′) or primer C (5′-GCARTGYAANACRTGNGARTC-3′), which are antisense-strand primers encoding amino acids 16–21 (DSHVLH) with three extra nucleotides at the 5′ termini to increase annealing stability. PCR with Taq polymerase was performed for 35–45 cycles, until product bands were apparent on agarose gel electrophoretic analysis. For the first two cycles of PCR, the reannealing step was performed at 37°C for 2 min; for the remainder of the cycles, reannealing was at 50°C for 1 min. Multiple product bands were observed in each reaction. Portions of the gel containing bands of approximately the expected size (66 bp) were reamplified with the same primer pair. The DNA products were cloned into vector pCR II (Invitrogen, San Diego, CA) according to the instructions of the manufacturer. Three clones were sequenced and were found to encode, in one reading frame, the expected canine MGDF sequence, residues 1–21. In this way, unique canine MGDF cDNA sequence was obtained spanning the region from the third nucleotide of codon 6 through the third nucleotide of codon 15. One of these clones served as template for preparation of a labeled canine MGDF cDNA probe.

Unique sequencing primers were designed based on this sequence and used with the adapter portion of the random primer in 3′ rapid amplification of cDNA ends (RACE) to generate clones, some of which extended the open reading frame from the canine kidney cDNA described above. Random-primed adult murine liver cDNA from mouse strains C57BL and DBA was amplified with pairs of primers based on

the canine sequence and 3′ RACE was performed, as described for the cloning of canine cDNA.

Isolation of cDNA Coding for MGDF

RNA was isolated from human fetal liver (International Institute for the Advancement of Medicine) by lysis of tissue in 5.5 M guanidinium thiocyanate and purification via CsTFA (Pharmacia) centrifugation. Polyadenylated RNA was selected using oligo(dT)25 Dynabeads (Dynal, Skoyen, Norway) according to the instructions of the manufacturer. Double-stranded cDNA was produced from this RNA using the SuperScript plasmid system for cDNA synthesis (Life Technologies, Gaithersburg, MD), except that a different linker–adapter (oligo 1, 5′-TTGGTGTGCACTTGT-3′; oligo 2, 5′-CACAAGTGCACCAACCCC-3′) was used. After size selection, this cDNA was directionally inserted into the BstXI and NotI sites of the mammalian expression vector pBCB, which is derived from the plasmid Rc/CMV (Invitrogen, San Diego, CA), comprising the pUC19 backbone, cytomegalovirus promoter, and BGH polyadenylation site and a modified multiple cloning site. The ligated DNA was electroporated into electrocompetent bacterial strain DH 10B (Life Technologies, Gaithersburg, MD).

Filter replicas of the human fetal liver library were hybridized to radioactively labeled canine MGDF N-terminal cDNA PCR product at 64°C for 18 hr (5 × SSPE, 2 × Denhardt's, 0.05% sodium pyrophosphate, 0.5% SDS, 100 μg/ml yeast tRNA lysate, and 100 μg/ml denatured salmon sperm DNA). Filters were washed at 64°C in 5 × SSPE, 0.5% SDS and exposed overnight. One clone hybridizing to this probe contained a 1.35 kb insert and was further analyzed.

Expression of Human MGDF cDNA Clones

Purified DNA from MGDF cDNA clones was transfected into 293 EBNA cells (Invitrogen, San Diego, CA). DNA (1.5 μg) was mixed with 7.5 μl lipofectamine (Life Technologies, Gaithersburg, MD) in 100 μl of serum-free DMEM. After a 20 min incubation at room temperature, the DNA–lipofectamine mixture was added to 5 × 10^5 cells/well (24-well square Greiner plates) in 400 μl of DMEM, 1% serum (Fetal Clone II) and incubated for 6 hr at 37°C; 500 μl of DMEM, 20% serum (Fetal Clone II) was added to the cells. The medium was aspirated 16 hr later, and 500 μl of DMEM, 1% serum (Fetal Clone II) was added. The conditioned media were collected 72 hr later and centrifuged through a 0.22 μm spin–filter. The conditioned media were assayed for MGDF biological activity.

Acknowledgments

Correspondence should be addressed to R. A. B. We thank L. Souza and B. Altrock for their support, J. Longyear and J. Bennett for preparation of the manuscript, and J. Keysor for preparation of the figures. We also thank T. Strickland, M. Rohde, B. Kenney, and J. Egrie for helpful discussions.

Received June 10, 1994.

References

Bazan, J. F. (1990). Structural design and molecular evolution of a cytokine receptor superfamily. Proc. Natl. Acad. Sci. USA *87*, 6934–6938.

Chomczynksi, P., and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. Anal. Biochem. *162*, 156–159.

DeClerck, Y. A., Yean, T.-D., Lu, H. S., Ting, J., and Langley, K. L. (1991). Inhibition of autoproteolytic activation of interstitial procollagenase by recombinant metalloproteinase inhibitor MI/TIMP-2. J. Biol. Chem. *266*, 3893–3899.

Devereux, J., Haeberli, P., and Smithies, O. (1984). A comprehensive set of sequence analysis programs for the VAX. Nucl. Acids Res. *12*, 387–395.

Erickson-Miller, C. L., Parchment, R. E., Zhang, Z. G., Ling, S. G., Ji, H., Dai, W., Tang, P. H., and Murphy, M. J. (1992). Purification of human megakaryocyte colony-stimulating factor (Meg-CSF) from urine of aplastic anemia patients. In Blood Cell Growth Factors: Their Present and Future Use in Hematology and Oncology, M. J. Murphy, ed. (Dayton, Ohio: AlphaMed Press), pp. 204–220.

Erickson-Miller, C. L., Ji, H., Parchment, R. E., and Murphy, M. J. (1993). Megakaryocyte colony-stimulating factor (Meg-CSF) is a unique cytokine specific for the megakaryocyte lineage. Br. J. Haematol. *84*, 197–203.

Evatt, B. L., Shreiner, D. P., and Levin, J. (1974). Thrombopoietic activity of fractions of rabbit plasma: studies in rabbits and mice. J. Lab. Clin. Med. *83*, 364–371.

Greenberg, S. M., Kuter, D. J., and Rosenberg, R. D. (1987). *In vitro* stimulation of megakaryocyte maturation by megakaryocyte stimulatory factor. J. Biol. Chem. *262*, 3269–3277.

Greenberger, J. (1983). Demonstration of permanent factor-dependent multipotential (erythroid/neutrophil/basophil) hematopoietic progenitor cell lines. Proc. Natl. Acad. Sci. USA *80*, 2931–2936.

Hendrie, P. C., Miyazawa, K., Yang, Y.-C., and Langefeld, C. D. (1991). Mast cell growth factor (c-*kit* ligand) enhances cytokine stimulation of proliferation of the human factor-dependent cell line, M07e. Exp. Hematol. *19*, 1031–1037.

Hill, R., and Levin, J. (1986). Partial purification of thrombopoietin using lectin chromatography. Exp. Hematol. *14*, 752–759.

Hill, R. J., Levin, J., and Levin, F. C. (1992). Correlation of *in vitro* and *in vivo* biological activities during the partial purification of thrombopoietin. Exp. Hematol. *20*, 354–360.

Hirano, T., Taga, T., Nakano, N., Yasukawa, K., Kashiwamura, S., Shimizu, K., Nakajima, K., Pyun, K. H., and Kishimoto, T. (1985). Purification to homogeneity and characterization of human B-cell differentiation factor (BCDF or BSF-2). Proc. Natl. Acad. Sci. USA *82*, 5490–5494.

Hirano, T., Yasukawa, K., Harada, H., Taga, T., Watanabe, Y., Matsuda, T., Kashiwamura, S.-I., Nakajima, K., Koyama, K., Iwamatsu, A., Tsunasawa, S., Sakiyama, F., Matsui, H., Takahara, Y., Taniguchi, T., and Kishimoto, T. (1986). Complementary DNA for a novel human interleukin (BSF-2) that induces B lymphocytes to produce immunoglobulin. Nature *324*, 73–76.

Ikebuchi, K., Wong, G. G., Clark, S. C., Ihle, J. N., Hirai, Y., and Ogawa, M. (1987). Interleukin 6 enhancement of interleukin 3-dependent proliferation of multipotential hemopoietic progenitors. Proc. Natl. Acad. Sci. USA *84*, 9035–9039.

Ishibashi, T., Kimura, H., Uchida, T., Kariyone, S., Friese, P., and Burstein, S. A. (1989). Human interleukin 6 is a direct promoter of maturation of megakaryocytes *in vitro*. Proc. Natl. Acad. Sci. USA *86*, 5953–5957.

Jacobs, K., Shoemaker, C., Rudersdorf, R., Neil, S. D., Kaufman, R. J., Mufson, A., Seehra, J., Jones, S. S., Hewick, R., Fritsch, E. F., Kawahakita, M., Shimizu, T., and Miyake, T. (1985). Isolation and characterization of genomic and cDNA clones of human erythropoietin. Nature *313*, 806–810.

Laemmli, U. K. (1970). Cleaving of structural proteins during the assembly of the head of bacteriophage T4. Nature *227*, 680–685.

Landau, N. R., and Littman, D. R. (1992). Packaging system for rapid production of murine leukemia virus vectors with variable tropism. J. Virol. *66*, 5110–5113.

March, C. J., Mosley, B., Larsen, A., Cerretti, D. P., Braedt, G., Price, V., Gillis, S., Henney, C. S., Kronheim, S. R., Grabstein, K., Conlon, P. J., Hopp, T. P., and Cosman, D. (1985). Cloning, sequence and expression of two distinct human interleukin-1 complementary DNAs. Nature *315*, 641–647.

Mazur, E., and South, K. (1985). Human megakaryocyte colony-stimulating factor in sera from aplastic dogs: partial purification, characterization and determination of hematopoietic cell lineage specificity. Exp. Hematol. *13*, 1164–1172.

Mazur, E. M., Basilico, D., Newton, J. L., Cohen, J. L., Charland, C., Sohl, P. A., and Narendran, A. (1990). Isolation of large numbers of enriched human megakaryocytes from liquid cultures of normal peripheral blood progenitor cells. Blood *76*, 1771–1782.

McDonald, T. P. (1975). Assay of thrombopoietin utilizing human sera and urine fractions. Biochem. Med. *13*, 101–110.

McDonald, T. P., Clift, R. E., Lange, R. D., Nolan, C., Tribby, E., and Barlow, G. H. (1975). Thrombopoietin production by human embryonic

kidney cells in culture. J. Lab. Clin. Med. *85*, 59–66.

Metcalf, D., Hilton, D., and Nicola, N. A. (1991). Leukemia inhibitory factor can potentiate murine megakaryocyte production *in vitro*. Blood *77*, 2150–2153.

Methia, N., Louache, F., Vainchenker, W., and Wendling, F. (1993). Oligodeoxynucleotides antisense to the proto-oncogene c-*mpl* specifically inhibit *in vitro* megakaryocytopoiesis. Blood *82*, 1395–1401.

Miyake, T., Kung, C. K. H., and Goldwasser, E. (1977). Purification of human erythropoietin. J. Biol. Chem. *252*, 5558–5564.

Odell, T. T., McDonald, T. P., and Detwiler, T. C. (1961). Stimulation of platelet production by serum of platelet-depleted rats. Proc. Soc. Exp. Biol. Med. *108*, 428–431.

Ogata, K., Zhang, Z. G., Abe, K., and Murphy, M. I. (1990). Partial purification and characterization of human megakaryocyte colony-stimulating factor (MEG-CSF). Int. J. Cell Cloning *8*, 103–120.

Paul, S. R., Bennett, F., Calvetti, J. A., Kelleher, K., Wood, C. R., O'Hara, R. M., Leary, A. C., Sibley, B., Clark, S. C., Williams, D. A., and Yang, Y. C. (1990). Molecular cloning of a cDNA encoding interleukin 11, a stromal cell-derived lymph opoietic and hematopoietic cytokine. Proc. Natl. Acad. Sci. USA *87*, 7512–7516.

Schmidt, J. A. (1984). Purification and partial biochemical characterization of normal human interleukin 1. J. Exp. Med. *160*, 772–787.

Skoda, R. C., Seldin, D. C., Chiang, M.-K., Peichel, C. L., Vogt, T. F., and Leder, P. (1993). Murine c-*mpl*: a member of the hematopoietic growth factor receptor superfamily that transduces a proliferative signal. EMBO J. *12*, 2645–2653.

Souyri, M., Vignon, I., Penciolelli, J.-F., Heard, J.-M., Tambourin, P., and Wendling, F. (1990). A putative truncated cytokine receptor gene transduced by the myeloproliferative leukemia virus immortalizes hematopoietic progenitors. Cell *63*, 1137–1147.

Tayrien, G., and Rosenberg, R. D. (1987). Purification and properties of a megakaryocyte stimulatory factor present both in the serum-free conditioned medium of human embryonic kidney cells and in thrombocytopenic plasma. J. Biol. Chem. *262*, 3262–3268.

Teramura, M., Kobayashi, S., Hoshino, S., Oshimi, K., and Mizoguchi, H. (1992). Interleukin-11 enhances human megakaryocytopoiesis *in vitro*. Blood *79*, 327–331.

Vignon, I., Mornon, J.-P., Cocault, L., Mitjavila, M.-T., Tambourin, P., Gisselbrecht, S., and Souyri, M. (1992). Molecular cloning and characterization of *MPL*, the human homolog of the v-*mpl* oncogene: identification of a member of the hematopoietic growth factor receptor superfamily. Proc. Natl. Acad. Sci. USA *89*, 5640–5644.

von Heijne, G. (1986). A new method for predicting signal sequence cleavage sites. Nucl. Acids Res. *14*, 4683–4690.

Wendling, F., Varlet, P., Charon, M., and Tambourin, P. (1986). Myeloproliferative leukemia virus, a retrovirus complex inducing an acute myeloproliferative leukemic disorder in adult mice. Virology *149*, 242–246.

Wendling, F., Penciolelli, J. F., Charon, M., and Tambourin, P. (1989). Factor-independent erythropoietic progenitor cells in leukemia induced by the myeloproliferative leukemia virus. Blood *73*, 1161–1167.

Wong, G., Witek, J. S., Temple, P. A., Wilkens, K. M., Leary, A. C., Luxenberg, D. P., Jones, S. S., Brown, E. L., Kay, R. M., Orr, E. C., Shoemaker, C., Golde, D. W., Kaufman, R. J., Hewick, R. M., Wang, E. A., and Clark, S. C. (1985). Human GM-CSF: molecular cloning of the complementary DNA and purification of the natural and recombinant proteins. Science *228*, 810–815.

Yamaguchi, N., Hattori, K., Oh-eda, M., Kojima, T., Imai, N., and Ochi, N. (1994). A novel cytokine exhibiting megakaryocyte potentiating activity from a human pancreatic tumor cell line HPC-Y5. J. Biol. Chem. *269*, 805–808.

Yang, Y. C., Ciarletta, A. B., Temple, P. A., Chung, M. P., Kovacic, S., Witek-Giannotti, J. S., Leary, A. C., Kriz, R., Donahue, R. E., Wong, G. G., and Clark, S. C. (1986). Human IL-3 (multi-CSF): identification by expression cloning of a novel hematopoietic growth factor related to murine IL-3. Cell *47*, 3–10.

GenBank Accession Number

The accession number for the sequence reported in this paper is U11025.